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Levels of EG-VEGF and VEGF in serum and in the follicular fluid on the day of oocyte retrieval and reproductive outcome among IVF patients

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Summary

The aim of this retrospective, observational, case control study was to investigate the correlation of endocrine gland-derived vascular endothelial growth factor (EG-VEGF) and vascular endothelial growth factor (VEGF) concentrations in serum and follicular fluid (FF) in women undergoing controlled ovarian hyperstimulation (COH) protocols and IVF/ICSI with the reproductive outcome. When the authors compared levels of EG-VEGF and VEGF in FF and serum, they did not observe differences in the group without pregnancy compared to the group of patients with clinical pregnancy (EG-VEGF, respectively, $p = 0.12$, $p = 0.94$, and $p = 0.16$ and VEGF $p = 0.16$, $p = 0.6$, and $p = 0.32$). The authors found lower levels of EG-VEGF in FF from large follicles in the group of patients with clinical pregnancy who miscarried compared to the group of patients who had no pregnancy ($p = 0.04$); when they compared this group with the group of patients who delivered a baby, there was no statistical significance. Lower levels of EG-VEGF in the follicular fluid from large follicles are negatively correlated with positive outcome of IVF, and correlate with miscarriage rates in patients after IVF.

Key words: Vascular endothelial growth factor; Endocrine gland-derived vascular endothelial growth factor; Controlled ovarian hyperstimulation; IVF/ICSI; Pregnancy.

Introduction

The follicular fluid (FF) contains protein and peptides from plasma or from metabolism of granulosa cells (GC), and constitutes microenvironment for the oocyte [1, 2]. The expression and secretion of some growth factors under controlled ovarian hyperstimulation (COH) protocols might be altered [3].

The endocrine gland–derived vascular endothelial growth factor (EG-VEGF) has similar biologic functions as vascular endothelial growth factor (VEGF). It induces tissue specific proliferation, migration, and fenestration in capillary endothelial cells and its expression is mainly restricted to steroidogenic glands [4]. EG-VEGF acts through its two G-protein–coupled receptors PRK1 and PRK2 (prokineticin receptor 1 and 2). Stimulation of these receptors activates calcium mobilization, leading to smooth muscle contractions and angiogenesis [5]. Ovary is the organ with the highest production of EG-VEGF, which is predominantly expressed by the granular and theca cells in follicles. The cellular localization and temporal and spatial expressions of EG-VEGF in ovarian tissue are complementarily correlated to VEGF [6, 7], suggesting that EG-VEGF and VEGF participate synergistically in ovarian angiogenesis of ovarian follicles or corpora lutea [8, 9].

In this study, the correlation between FF and serum levels of EG-VEGF, VEGF, and clinical pregnancy after IVF in COH cycles was investigated. FF and serum levels EG-VEGF and VEGF were measured in patients under both COH protocols.

Materials and Methods

This observational retrospective case control study was conducted in Novum, Fertility Clinic (Warsaw, Poland), where 117 infertile women were treated with IVF/ICSI. Patients were enrolled from January 2012 to February 2015. The study was approved by the Bioethical Committee of Central Clinical Hospital of Interior in Warsaw (Poland) (67/2011) and an informed consent for the collection and storage of serum, FF, which would not affect the IVF/ICSI procedure, was obtained from all patients in this study.

The patients were divided retrospectively into two groups. The first group consisted of 48 patients who became pregnant after IVF procedure. In the second group (69 women) the pregnancy was not achieved in any case. Among all study groups there were patients in the GnRH agonist protocol (long protocol), in the GnRH antagonist protocol (short protocol), and in the natural cycle. The women's characteristics are presented in Table 1.

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Table 1. — *Patients' characteristics.*

Variable/means, 95% CI ± SD	Pregnancy group (n=48)	Non-pregnancy group (n=69)	*p*-value
Age (years)	34.06 (32.94−35.19) ± 3.88	35.8 (34.61−36.99) ± 4.95	0.11
BMI (kg/m^2)	22.37 (21.08−23.67) ± 4.46	22.48 (21.68−23.27) ± 3.31	0.51
Time of infertility (years)	3.8 (2.99−4.61) ± 2.78	3.83 (3.15−4.5) ± 2.82	0.89
Type of infertility % (95%CI)			
Primary	62.50 (48.8−76.19)	55.07 (43.3−66.8)	0.22
Secondary	37.50 (23.8−51.2)	44.93 (31.1−56.6)	0.19
Type of COH % (95%CI)			
Natural cycle	29.17 (16.3−42.1)	44.93 (31.1−56.6)	0.04*
Long protocol with GnRH agonist	45.83 (31.7−59.9)	28.99 (18.3−39.7)	0.03*
Short protocol with GnRH antagonist	25.00 (12.75−37.2)	26.09 (16.4−37.4)	0.45
IVF % (95%CI)			
I	64.58 (51.1−78.1)	42.03 (30.3−53.6)	0.007*
II	20.83 (15.6−41)	24.64 (14.4−37.7)	0.30
III or more	14.58 (4.6−24.6)	33.33 (22.2−44.4)	0.01*
FSH (mIU/ml)	7.48 (6.3−8.65) ± 4.06	7.77 (7.01−8.52) ± 3.12	0.33
AMH (ng/ml)	2 (1.11−2.89) ± 2.1	2.07 (1.23−2.91) ± 2.83	0.23
FF VEGF (large follicles) (pg/ml)	1029.09 (917.69−1140.48) ± 379.38	1096.05 (1006−1186.09) ± 360.48	0.32
FF VEGF (small follicles) (pg/ml)	736.47 (610.1−862.85) ± 384.48	773.47 (658.8−888.14) ± 399.24	0.6
Serum VEGF (pg/ml)	135.94 (113.89−157.98) ± 75.92	115.87 (102.9−128.83) ± 53.97	0.16
FF EG-VEGF (large follicles) (pg/ml)	3372.53 (3084.75−3660.31) ± 980.15	3683.89 (3419.1−3948.68) ± 1060.03	0.16
FF EG-VEGF (small follicles) (pg/ml)	2222.55 (1928.08−2517.03) ± 895.9	2187.51 (1905.75−2469.27) ± 980.93	0.94
Serum EG-VEGF (pg/ml)	14.1 (11.02−17.19) ± 10.63	18.57 (15.41−21.72) ± 13.13	0.12

**Statistical significance p < 0.05.*

In patients in the natural cycle group, after at least one dominant follicle reached the diameter of 17 mm, hCG was administered. In patients treated with COH, a flexible GnRH antagonist protocol or a long GnRH agonist protocol was used for controlled ovarian stimulation, as previously described [10]. Shortly, in GnRH agonist long protocol, after pituitary suppression with triptorelin, which started in the midluteal phase of preceding cycle, ovarian hyperstimulation was performed by administration of both menotropin and rFSH. The starting doses ranged from 75 to 150 IU/day in the case of menotropin and between 37.5 IU and 225 IU/day for rFSH. The GnRH antagonist (short protocol) multiple-dose protocol involved the daily administration of 0.25 mg GnRH antagonist cetrorelix acetate from the day the dominant follicle was about 12-13 mm in diameter until hCG administration. Follicle size was monitored serially by transvaginal ultrasound (6.5 MHz transvaginal probe). The gonadotropin dose was adjusted according to the patient's response, measured by sequential transvaginal ultrasonography and the serum E2 levels. The starting doses of menotropin ranged from 75 to 150 IU/day and between 37.5 IU and 225 IU/day for rFSH. HCG was administered in a dose of 5,000 IU in the presence of at least three follicles of ≥ 17 mm in diameter along with the increase in serum E2 concentration.

Transvaginal ultrasound-guided oocyte retrieval was performed 36 hours after hCG administration. On a day of oocyte retrieval, blood sample of 15 ml was collected from a patient, and serum after preparation was stored at -80°C for further analysis.

The FF was aspirated separately from each follicle during oocyte retrieval. Any follicle aspirates that were not clear or contaminated with blood were discarded. Follicles were divided into two groups depending on the size measured by ultrasonography on the day of oocyte retrieval; small follicles smaller or equal than 17 mm, large follicles larger than 17 mm in diameter. FF from follicles from a single patient were pooled depending on the follicle size. FF was immediately centrifuged for 15 minutes at 1,500 rpms and the supernatants were stored.

Intracytoplasmic sperm injection was performed in all cases. Single embryo transfer was performed five days after oocyte aspiration. Micronized progesterone was administered vaginally and sublingually from the day of oocyte pick-up and continued for six to eight weeks if pregnancy was confirmed.

On the day of oocyte retrieval, after obtaining a written consent from the patient, blood was collected from the peripheral vein. FF was obtained from small (≤ 17 mm) and from large follicles (> 17 mm). In some cases, especially in patients from the control group, FF was collected only from large follicles, because of the lack of small stimulated follicles,. Aliquots of the individually pooled FF, as well as serum samples, were stored at 70°C until the assay.

The concentration of VEGF, EG-VEGF in serum and FF was determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits. The samples were prepared and tested in duplicate according to the manufacturers' instructions. The concentrations were expressed as pg/ml. The assay sensitivity was five pg/ml. The inter- and intra-assay coefficients of variation were < 7 and < 4.5%, respectively.

Statistical analyses were performed with STATISTICA 10.0 software. A $\alpha = 0.05$ error was considered to be significant for all comparisons. The Shapiro-Wilk test was used to verify normality. All continuous variables were non-normally distributed. They were presented as median and interquartile range. Categorical variables were presented as number (percentage). χ^2 tests and taper factors were used for the correlation of categorical variables. The Mann-Whitney U-test, Kruskal-Wallis, and multiple repetition tests compared continuous variables and Friedman's test, while categorical by Fisher's test.

Results

There were no statistical differences between VEGF and EG-VEGF levels in serum and follicular fluids from small and large follicles when comparing both groups with pregnancy and without pregnancy after IVF (Table 1).

When the authors isolated a group of patients who had clinical pregnancy and who miscarried from the group with clinical pregnancy and compared them with the group without pregnancy after IVF, levels of EG-VEGF in FF from large follicles were statistically higher in the group without pregnancy compared to the group of patients with clinical pregnancy and miscarriage (2,448 pg/ml *vs*. 180 pg/ml respectively) (p = 0.04, Figure 1). Such an observation was not observed for FF from small follicles (1,440 pg/ml *vs*. 156 pg/ml) (p = 0.29) and in serum (2,817 pg ml *vs*. 264 pg/ml) (p = 0.16). There was no statistical correlation between VEGF concentration in FF from large follicles in both groups (2,398 pg/ml *vs*. 230 pg/ml) (p = 0.28), neither in FF from small follicles (1,379 pg/ml *vs*. 217 pg/ml) (p = 0.68) nor in serum (2,683.5 pg/ml *vs*. 397.5 pg/ml) (p = 0.52) (no pregnancy group *vs*. pregnancy group with miscarriage, respectively).

When the authors compared the group of patients with pregnancy to the group of patients with pregnancy who had a miscarriage, there were also lower levels of EG-VEGF in FF but without statistical significance (992 pg/ml *vs*. 136 pg/ml) (p = 0.12, Figure 2), which may result from a small sample size (nine patients with miscarriage *vs*. 39 pregnant women who delivered a baby).

In all of the present patients the authors found linear correlation between EG-VEGF levels in FF from small and large follicles and the serum (the higher EG-VEGF level in FF from small follicles, the higher EG-VEGF level in large follicles, and subsequently in the serum). In all patients, EG-VEGF level from FF from large follicles was correlated with VEGF level in both large, as well as in small follicles. The VEGF and EG-VEGF concentration in serum and FF was not correlated with estradiol levels.

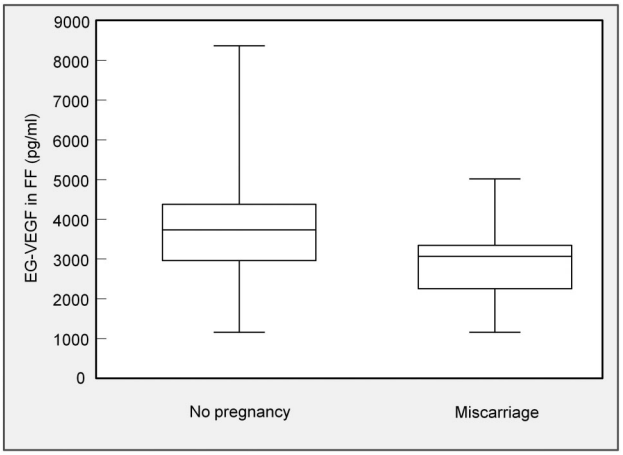

Figure 1. — Concentration of EG-VEGF in FF (pg/ml) from large follicles. Group without pregnancy *vs*. clinical pregnancy and miscarriage group (p = 0.04).

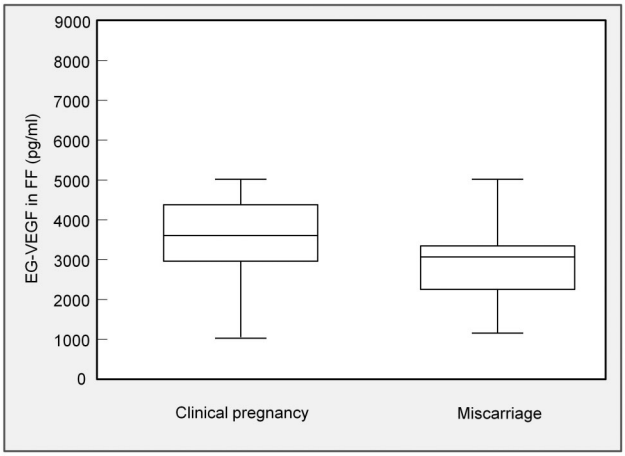

Figure 2. — Concentration of EG-VEGF in FF (pg/ml) from large follicles. Group of patients with pregnancy to the group of patients with pregnancy who had miscarriage (p = 0.12).

Discussion

The data presented provide evidence that concentration of EG-VEGF or VEGF in FF on the day of oocyte retrieval or in serum cannot be used as a prognostic marker of conception rates in ART cycles. We might postulate that lower levels of EG-VEGF in FF might influence the quality of oocyte and might correlate with miscarriage rates in patients after IVF compared to the group of patients without pregnancy.

There is some proof that a higher level of VEGF might influence the quality of oocyte, the quality of embryo or affect pregnancy. The level of VEGF in FF from non-pregnant patients after IVF was shown to be higher compared to patients who achieved pregnancy [11]. Also the higher level of VEGF in FF is significantly associated with diminished pregnancy potential in IVF cycles [12]. Higher levels of VEGF in FF is negatively correlated with embryo quality as determined by morphology scoring on day 3 of in vitro culture [13].

It was also proven that concentration of VEGF in FF at the time of oocyte retrieval depends on patients' age and is higher in older woman [14]. In the present study the authors did not find a correlation between patients' age and VEGF or EGVEGF concentration in FF. They also identified no correlation between pregnancy group or BMI, FSH or AMH (Table 1).

EG-VEGF was first characterized and sequenced in 2001 [15]. Despite the fact that this protein does not show any structural homology to the VEGF family, it displays sev-

eral striking biological similarities to VEGF, including the ability to induce fenestration in the target cells. Angiogenic action of EG-VEGF appeared to be restricted to endothelial cells derived from endocrine tissues [15]. In endothelial cells isolated from steroidogenic glands, EG-VEGF promotes proliferation, survival, and chemotaxis [5]. Inactivation of VEGF results in dramatic suppression of corpus luteum development, with less affected endothelial cell growth associated with follicular development. EG-VEGF potentially contributes to such VEGF independent angiogenesis [15].

Both VEGF and EG-VEGF act additively or in a cooperation on adrenal cortex derived capillary endothelial cells (ACE) in inducing fenestration, while no effect was observed in the absence of VEGF or EG-VEGF. The fenestrae are highly permeable to fluid and small solutes and are thought to facilitate large exchange of materials between interstitial fluid and plasma [7]. The role of EG-VEGF at the mid- to late luteal phase is the regulation of vascular permeability to enhance transport of LDLs into the luteal cells and secretion of progesterone and other luteal products into the blood stream. This may allow corpus luteum to respond to hCG in early pregnancy or over-respond in patients with OHSS [9]. It was also demonstrated that hCG stimulates synthesis of VEGF and EG-VEGF [9]. In human placenta EG-VEGF acts angiogenically on selective endothelial tissue human placenta endothelial cells (HPEC), i.e. microvascular cells covering the fetal capillaries of chorionic villi [16] and increase the permeability and the paracellular transport in these cells. The present authors hypothesize that EG-VEGF potentially acts also outside the endocrine glands on endothelial cells and cooperates in this field with VEGF. In literature there is only one paper where EG-VEGF was measured in patient serum and FF during COH, with GnRH agonist protocol, and compared with OHSS risk [17]. Its authors showed that EG-VEGF and VEGF levels were much higher in FF than in serum. They also discovered a highly significant positive correlation between FF level of EG-VEGF with FF VEGF, suggesting that they act synergistically in follicle development.

Altogether, the results of the present study may provide a new approach to the potential role of EG-VEGF in ovarian response and oocyte maturation, which can also influence the embryo quality and chances for achieving a clinical pregnancy.

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